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Bioacessibilidade *in vitro* de ácidos orgânicos em Vinhos Verdes

In vitro bioacessibility of organic acids in Vinhos Verdes

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Resumo

Introdução: O Vinho Verde é exclusivamente produzido na Região Demarcada dos Vinhos Verdes, em Portugal. A fermentação maloláctica que ocorre na produção destes vinhos pode implicar diferenças na concentração de ácidos orgânicos, que, consoante alguns estudos pode despoletar uma resposta gástrica provocando desconforto a indivíduos com patologias gastrointestinais.

Objectivo: Este estudo teve como objectivo a comparação entre Vinhos Verdes e os restantes pela quantificação do seu teor de ácidos orgânicos e avaliação da sua bioacessibilidade.

Materiais e Métodos: Dez amostras de vinho foram analisadas por cromatografia gasosa acoplada à espectrometria de massa em três momentos da investigação: após abertura e após as fases gástrica e intestinal do processo digestivo, simulado por um modelo standardizado de digestão *in vitro*.

Resultados e Discussão: Inicialmente os valores de ácido láctico e succínico são menores em Vinhos Verdes e maiores em maduros tintos, ao contrário do ácido málico, com valores mais elevados nos primeiros. Os teores dos restantes ácidos são semelhantes entre os diversos tipos de vinho. Após a digestão, ácidos orgânicos mais simples aumentam a sua concentração, sugerindo degradação ou precipitação, devido a variações de pH, dos mais complexos. O ácido málico dissipa-se em todos os vinhos à excepção dos Verdes, sendo improvável o desencadeamento de uma resposta gástrica uma vez que a concentração remanescente é diminuta.

Conclusões: As diferenças entre Vinhos Verdes e restantes vinhos não parecem, sobretudo após o processo digestivo, suficientes para exercer efeitos distintos na

acidez gástrica. Todavia, mais estudos podem ser desenvolvidos de modo a avaliar o efeito da microbiota intestinal na metabolização destes compostos.

Palavras-Chave: Ácidos orgânicos, Bioacessibilidade, Cromatografia gasosa acoplada à espectrometria de massa, Vinhos.

Abstract

Background: Vinho Verde (Verde wine) is exclusively produced in the Demarcated Vinho Verde Region in Portugal. The malolactic fermentation that occurs in this wine's production may imply differences in organic acid levels, which according to some studies can trigger a gastric response and cause discomfort to subjects with gastrointestinal disorders.

Aim: This study aimed a comparison between Vinho Verde and other types of wine by the quantification of its organic acids content, followed by their bioaccessibility assessment.

Materials and methods: Ten samples of wine were analysed by gas chromatography-mass spectrometry at three moments of the experiment: right after opening and after gastric and intestinal phase of digestion. To evaluate the concentration after the last two phases referred, an *in vitro* standardised digestion model was applied.

Results and Discussion: Initially, lactic and succinic acid values are lower in Vinho Verde and higher in red wine, unlike malic acid, with higher values in the first ones. The remaining acids concentration is nearly in the same range between wines. Smaller, simpler acids concentration increased after digestion suggesting degradation or precipitation, due to pH variation, of bigger ones. Malic acid disappears in all wines except in Vinho Verde, although is unlikely to trigger a gastric response since the remaining amount is very low.

Conclusions: The differences between Vinho Verde and the other wines do not seem, especially after the digestive process, enough to have different effects on gastric acidity. Still, more studies can be performed to evaluate the effect of intestinal microbiota in these compound's metabolization.

Keywords: Bioaccessibility, Gas Chromatography-Mass Spectrometry, Organic acids, Wines.

Abbreviations and acronyms

CVRVV - Commission of Viticulture of the Region of Vinho Verde

GC-MS - Gas Chromatography/Mass Spectrometry

p.a. - pro analysis

MSTFA - N-methyl-N-(trimethylsilyl)trifluoroacetamide

NH4I - Ammonium iodide

DTE - 1,4-dithioerythritol

MTBSTFA - N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide

HCl - Hydrochloric acid

TEA - Triethylamine

KCl - Potassium chloride

KH_2PO_4- Potassium phosphate monobasic

$NaHCO_3$ - Sodium bicarbonate

NaCl - Sodium chloride

NaOH - Sodium hydroxide

$MgCl_2(H_2O)_6$ - Magnesium chloride, 6-hydrate

$(NH_4)_2CO_3$ - Ammonium carbonate

$CaCl_2(H_2O)_2$ - Calcium chloride, dihydrate

USP - United States Pharmacopeia

HPLC - High Performance Liquid Chromatography

$MgSO_4$ - Magnesium sulfate

VW - White Vinho Verde

VR - Red Vinho Verde

S - Sparkling wine

WW - White wine

RW - red wine

SIM - Selected Ion Monitoring

RCC - Correlation coefficient values of calibration curves

LOD - Limit of detection

cv - variation coefficient

RRC - Correlation coefficient values of recovery curves

Index

Introduction

Wine is defined as “the product obtained exclusively by the total or partial alcoholic fermentation of fresh grapes, whether or not pressed, or of fresh grape must, with an alcoholic grade not less than 8,5%, which must be drinkable and fit for consumption, according to the law.”(1)

Portugal, one of the largest wine exporters, with 3 million hectoliters exported in 2017, leaders in the consumption segment, with an average of 51.4 liters of wine per person per year.(2)

Vinho Verde (Verde wine) is exclusively produced in the Demarcated Vinho Verde Region, in Portugal, only from native grape’s varieties of the region. Such conditions preserve the typical and differentiating flavours, affirming Vinho Verde as unique worldwide. Naturally light and fresh, the unique set of wines offered by this region constitute a Denomination of Controlled Origin by the Commission of Viticulture of the Region of Vinho Verde (CVRVV), with each bottle bearing a guarantee seal.

The Demarcated Region of Vinhos Verdes had its quality and genuineness officially recognized by the attribution of the demarcation of production area, making it, geographically, the largest denomination in Portugal and one of the oldest wine-growing regions of the world, involving thousands of producers that make it a solid contribution to the economy and development not only of the Minho region but also of the whole country.

Extending throughout the north-west of Portugal, in the agricultural region known as Entre-Douro-e-Minho (between Douro and Minho rivers), this region is marked by an extreme influence of the Atlantic Ocean, a phenomenon reinforced

by the valley's orientation of the main rivers, that running from source to west facilitate the penetration of the sea winds.[3]

One of the differences in the production of Vinho Verde is the malolactic fermentation process, which unlike other wines, does not occur. This process takes place in many wines, generally 2-3 weeks after completion of alcoholic fermentation and consists in the conversion of L-malic acid to L-lactic acid by bacteria, mainly *Oenococcus oeni* (formerly *Leuconostoc oenos*). Its role in winemaking is the deacidification by the conversion of a dicarboxylic acid to a softer monocarboxylic acid, the microbial stability of wine and the wine aroma and flavor modifications.[4-6] This may imply differences in the organic acid levels between Vinho Verde and the others.

Organic acids are compounds with acidic properties and carboxylic acids, whose acidity is related to the carboxyl group (-COOH), are the most common. They occur naturally in food and are important indicators of various metabolic and biochemical processes.[7] In wines, they may be present in grapes or they may occur from the processes of alcoholic and malolactic fermentation, at the time of its production. We know that citric, malic and tartaric have its origin in grapes while acetic, lactic and succinic are products of biological reactions.[8]

Gas Chromatography/Mass Spectrometry (GC/MS) is a technique used in bromatological analysis capable of identifying and providing detailed information about molecular structures, being considered a valid alternative to other methods of carboxilic acids determination in wines.[9]

The ingestion of alcoholic beverages can stimulate gastric acid secretion and therefore may cause discomfort to subjects with gastrointestinal disorders.[10] Several studies show that some organic acids, like malic, succinic and maleic, play

an important role in gastric response increase.[11-13] *In vitro* digestion models are widely used to test and create new hypothesis regarding the digestive process as they study bioaccessibility - substance fraction that will be available for absorption in the intestine, after digestion.[14] For its simplicity it can be easily applied to matrices like wine.

Aim

The aim of this study was the comparison between Vinho Verde and other types of wine by the quantification of its organic acids content, followed by a bioaccessibility assessment with the implementation of a standardised digestion model.

Experimental

1. Reagents and Materials

All organic acids (p.a.) were purchased from Fluka, Aldrich and Alfa Aesar, and the stock solutions were prepared with ethanol (Carlo Era, ≥99.9%). The derivatization reagents used were N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA, ≥98.5% purity) and its catalysts, ammonium iodide (NH_4I, ≥99%) and 1,4-dithioerythritol (DTE, ≥99%), mixing 1mL of MSTFA with 8mg of NH4I and 4mg of DTE and N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA, >97%), all obtained from Sigma-Aldrich.

Ion exchange resin (Dowex 50W - X8) was bought from Sigma and activated with methanol, water, HCL 0.1M, water and the left to dry to the next day. Triethylamine (TEA, 99.8%) was purchased from Fisher Chemical and 2,2,4-Trimethylpentane (iso-Octan, ≥99%) from Sigma-Aldrich.

The reagents used to prepare the digestion fluids included KCl, KH_2PO_4, $NaHCO_3$, NaCl, HCl, NaOH (all bought from Merck), $MgCl_2(H_2O)_6$ (Riedel-de Haen), $(NH_4)_2CO_3$, $CaCl_2(H_2O)_2$ (Sigma), α-amylase from *Aspergillus oryzae* (Sigma, ~1.5U/mg), pepsin from porcine stomach mucosa (Sigma, ≥400 units/mg protein) bile porcine extract (Sigma), lipase from porcine pancreas (Sigma, type II), pancreatin from porcine pancreas (Sigma, meets USP testing specifications). The reagents used for organic acids extraction after this process included acetonitrile (Sigma, HPLC grade 99%) and $MgSO_4$ (Merck).

Ultra high purity Helium for GC-MS was obtained from Gasin.

All solvents and reagents were of analytical grade.

2. Sampling

A total of 10 wine samples (and their duplicates), comprising 5 wines from Vinho Verde region, 4 white Vinhos Verde (VW) and 1 red Vinho Verde (VR), and 5 wines not from Vinho Verde region, 1 sparkling wine (S), 2 white wines (WW) and 2 red wines (RW), were supplied from a wine company from Vinho Verde region.

3. pH and Alcoholic grade measurement

pH was evaluated using a glass electrode, and alcoholic grade was obtained after a distillation process and further measurement with an alcoholometer. (Attachment A) All samples were stored at 4ºC until further analysis.

4. GC-MS Equipment

The analysis were performed using a gas chromatograph 6890N (Agilent, Little Falls, DE, USA) equipped with a Combi-PAL autosampler (CTC Analytics, Zwingen, Switzerland) and an electronically controlled split/splitless injector port, interfaced to a single quadruple inert mass selective detector (5975B, Agilent) with

electron ionization chamber. GC separation was performed on a DB-5MS column (30m x 0,25mm I.D. x 0,25µL film thickness; J&W Scientific, Folsom, CA, USA). Helium was the carrier gas with a constant flow of 2mLmin^{-1}

5. Organic acids in wine: analysis

5.1 Sample Preparation

To 250µL of wine was added 2250µL of ethanol and 250mg of ion exchange resin, previously activated with methanol and water followed by the addition of hydrochloric acid 0.1M and filtration with water.

5.2 Derivatization Procedure

For the quantification of citric, lactic, oxalic, succinic, maleic, malic, and tartaric acids, 100 µL of the previous preparation were added with 20µL of internal standards benzylmalonic acid (0.5 gL^{-1}) and quinic acid (0.5gL^{-1}). The sample was evaporated until totally dry under nitrogen stream and then 100µL of MSTFA were added in order to proceed to the derivatization process in the microwave (1200W) for 3 minutes.

For quantification of acetic acid, 250µL were collected, added with 20µL of formic acid (0.5gL^{-1})[15] and 5µL TEA and reduced to 50µL under nitrogen stream. For the derivatization process these were added with 60µL of 2,2,4-Trimethylpentane and 20µL of MTBSTFA[7] and placed in the microwave (1200W) for 3 minutes.

5.2.1 Chromatographic conditions and detection - citric, lactic, oxalic, succinic, maleic, malic, and tartaric acids

The injection of 1µL was made in split mode (1:20) (purge-off time, 60s) at 250°C. The oven temperature program was as follows: 70°C held for 2 min, ramped to 80 at 8°Cmin^{-1}, ramped to 150 at 4ºCmin^{-1}, ramped to 290 at 18ºCmin^{-1},

held for 2.47min. Total run time was 31 min. The MS transfer line was held at 280 °C. Mass spectrometry parameters were set as follows: electron ionization with 70 eV energy; ion source temperature, 230°C and MS quadrupole temperature, 150°C. The quantification was made in Full Scan and the ions used to identification are in Attachment B. Agilent MSD ChemStation (version E.02021431) was used for data collection/processing and GC–MS control.

5.2.2 Chromatographic conditions and detection - acetic acid

The injection of 1µL was made in splitless mode (purge-off time, 45s) at 250°C. The oven temperature program was as follows: 70°C held for 2 min, ramped to 80 at 8°Cmin-1, ramped to 290 at 28ºCmin-1, held for 15s. Total run time was 11 min. The MS transfer line was held at 280 °C. Mass spectrometry parameters were set as follows: electron ionization with 70 eV energy; ion source temperature, 230°C and MS quadrupole temperature,150°C. The quantification was made in selected ion monitoring (SIM), m/z 75 and 117 for acetic acid and 75, 103 for formic. The ions m/z 103 and 117 were used for quantification of formic acid and acetic acid, respectively. Agilent MSD ChemStation (version E.02021431) was used for data collection/processing and GC–MS control.

6. In vitro digestion model

6.1 Organic acids bioaccessibility

Wine samples were digested in quadruplicate with three digestion fluids (salivary, gastric, intestinal) according to a standardised model described by Minekus *et al*[14]. Three types of wines were tested: a white Vinho Verde, a white wine and a red wine.

For each sample, 5mL were digested in falcon tubes at 37ºC using a Rotary Tube Mixer with Disc (25rpm; LSCI, Portugal) in an incubator (Genlab, UK).

Simulated digestion included: oral phase (5mL of sample, 3.5mL of salivar fluid and 0.5mL of α-amylase solution for 2min at pH 7), gastric phase (10mL of sample from previous phase, 7.5mL of gastric fluid and 1.6mL of pepsin solution for 2h at pH 3) and intestinal phase (10mL of sample from previous phase, 5.5mL of intestinal fluid, 2.5mL of pancreatin solution, 0.25mL of lipase solution and 1.25mL of bile solution for 2h at pH 7). The pH was adjusted with NaOH (1M) or HCl (1M). The reaction tubes were placed on ice in order to stop de digestion process and centrifuged for 5min at 4500xg. The samples were stored at 4ºC until the moment of analysis.

6.1.1 Sample Preparation

Organic acids were quantified at the end of gastric phase and at the end of the process. The extraction was performed accordingly to QuEChERS method[16] in which 1mL of sample was mixed with 1mL of acetonitrile, 400mg of $MgSO_4$ and 100mg on NaCl. Then, this mixture was shaken in a vortex and centrifuged for 7min at 4500xg.

6.1.2 Sample Analysis

The derivatization procedure of these samples occurred the same way as described in 5.2.

Results

The following figure and table show an example of a wine sample chromatogram and its peaks identification and the values obtained when measuring pH and alcoholic grade.

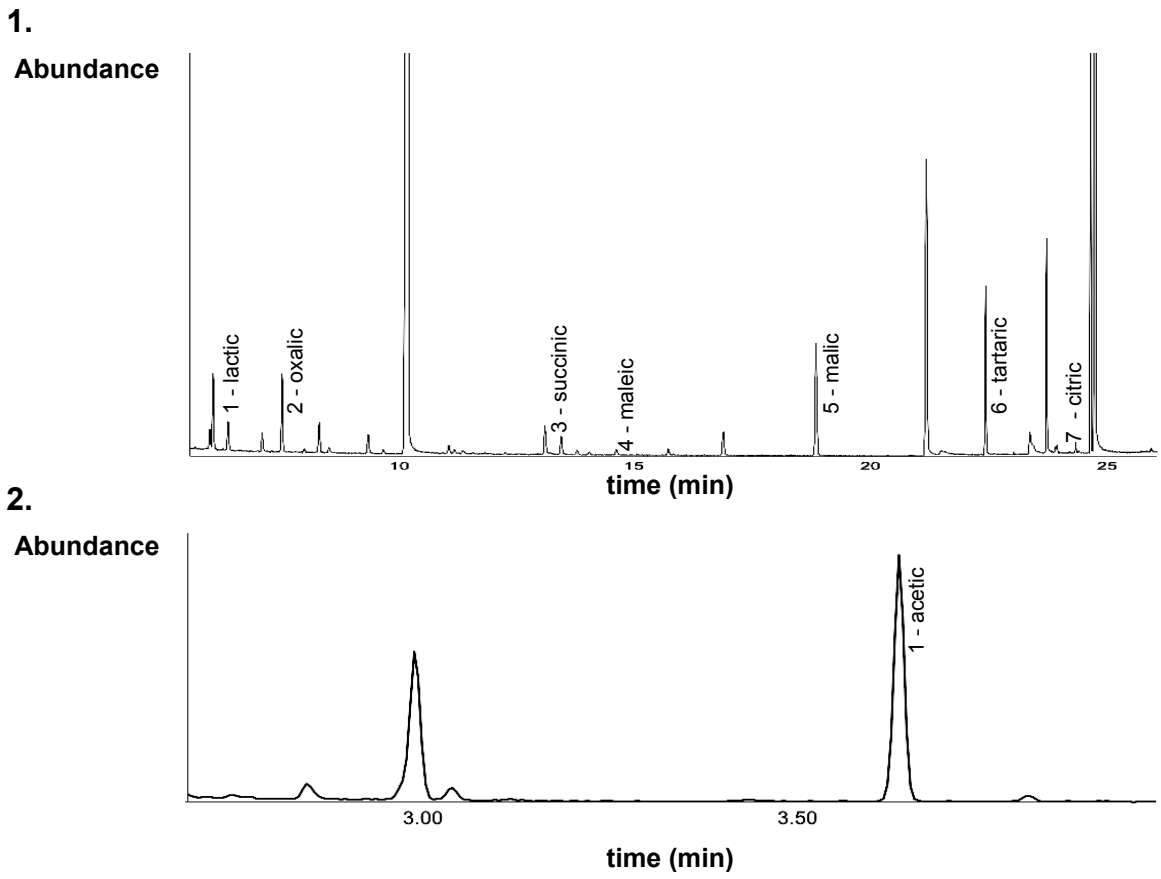

Figure 1. Chromatograms of a wine sample **(1)** 1 - lactic; 2 - oxalic; 3 - succinic; 4 - maleic; 5 - malic; 6 - tartaric; 7 - citric **(2)** 1- acetic

Table 1. pH and Alcoholic grade (% Vol) of 10 wine samples. **VW** - white Vinho Verde; **WW** - white wine; **S** - Sparkling wine; **VR** - red Vinho Verde; **RW** - red wine.

	pH	Alcoholic Grade (% Vol.)
VW	3,32	9,52
VW	3,55	12,3
VW	3,28	11,3
VW	3,21	11,54
WW	3,51	12,3
WW	3,27	12,4
S	3,57	12,2
VR	3,56	11,98
RW	3,67	12,9
RW	3,90	13,48

7. Analytical Procedure

7.1 Linearity

Calibration curves were constructed by analysing aqueous standard solutions of the 8 organic acids studied with increased amount of each acid, treated the exact same way as samples. Correlation coefficients were higher than 0.99 for all the 8 acids. (Table 2. RCC)[17, 18]

7.2 Detection Limit

The detection limit is defined as the lowest analyte concentration, that can be detected but not necessarily quantified, which provides a response in the detector significantly different than a blank sample. In this study it was calculated as a signal-to-noise ratio of 3. (Table 2. LOD) [17, 18]

7.3 Precision

The method's precision was evaluated by 6 analysis of the same sample under equal analytical conditions. The percentage of agreement, given by the variation coefficient ($CV = (\sigma / \bar{x}) * 100$) shown values below 20% for all except lactic acid. No results were obtained for maleic and oxalic acids. (Table 2. CV) [17, 18]

7.4 Recovery

Recovery curves were constructed by adding standard solutions with 6 increasing concentration levels to wine samples (pre-diluted with ethanol). These presented correlation coefficient values above 0.99 for lactic, succinic, acetic, maleic and tartaric acids and between 0.98 and 0.99 for citric and malic acids. (Table 2. RRC) [17, 18]

Table 2. Correlation coefficient values of calibration curves and recovery curves (RCC, RRC), limits of detection (LOD) and variation coefficient (CV)

	RCC	RRC	LOD	CV
ACETIC	0,999	0,998	0,007	11
CITRIC	0,998	0,985	0,001	3
LACTIC	0,996	0,990	0,004	22
MALEIC	0,994	0,994	0,031	-
MALIC	0,999	0,989	0,023	12
OXALIC	0,995	0,998	0,017	-
SUCCINIC	0,999	0,994	0,004	17
TARTARIC	0,999	0,998	0,004	9

8. Organic acids in wine: Quantification

Table 3 shows the results obtained from the analysis of wine sample before the digestion process. The amounts of organic acids obtained in these samples were comparable to the ones obtained in other studies.[8]

Table 3. Concentration (gL^{-1}) of organic acids in 10 wine samples. **VW** - white Vinho Verde; **WW** - white wine; **S** - Sparkling wine; **VR** - red Vinho Verde; **RW** - red wine.

	LACTIC	OXALIC	SUCCINIC	MALEIC	MALIC	TARTARIC	CITRIC	ACETIC
1. VW	1,266	1,069	0,288	0,090	2,186	1,305	0,372	0,258
2. VW	0,231	0,906	0,225	0,072	2,394	0,979	0,372	0,368
3. VW	0,215	0,524	0,250	0,064	1,790	1,331	0,380	0,392
4. VW	0,238	0,454	0,325	0,060	2,248	1,547	0,438	0,308
5. WW	0,246	0,547	0,206	0,051	1,607	1,966	0,250	0,398
6. WW	0,241	0,595	0,268	0,056	1,155	1,207	0,450	0,436
7. S	0,287	0,630	0,500	0,069	2,516	1,595	0,583	0,378
8. VR	2,220	0,725	0,617	0,050	0,312	2,082	0,191	0,351
9. RW	1,368	0,576	0,732	0,050	0,304	1,386	0,138	0,536
10. RW	2,168	0,794	0,834	0,048	0,278	1,227	0,042	0,378

Table 4 shows the amounts of each organic acid in 3 wine samples (white Vinho Verde, white wine and red wine) obtained after the simulated digestion process.

Table 4. Concentration (gL^{-1}) of organic acids in three types of wine after bioaccessibility assessment. **VW** - Vinho Verde (white); **WW** - white wine; **RW** - red wine; **I** - initial values; **GF** - gastric phase values; **IF** - intestinal phase values.

		LACTIC	OXALIC	SUCCINIC	MALEIC	MALIC	TARTARIC	CITRIC
VW	**I**	0,215	0,524	0,250	0,064	1,790	1,331	0,380
	GF	0,231	1,441	0,394	0,105	0,813	0,449	0,120
	IF	0,262	1,783	0,097	-	0,439	-	-
WW	**I**	0,241	0,595	0,268	0,056	1,155	1,207	0,450
	GF	0,251	1,689	0,404	0,104	0,597	0,441	0,131
	IF	0,269	0,909	0,098	-	-	-	-
RW	**I**	1,368	0,576	0,732	0,050	0,304	1,386	0,138
	GF	-	-	-	-	-	-	-
	IF	0,348	0,444	-	-	-	-	-

Discussion

Considering the initial amounts of organic acids in wines and comparing the Vinho Verde (white) and the red wines we can conclude: Lactic acid values are higher in the last ones (mean: $2,452gL^{-1}$) and lower in the first ones (mean: $0,4875gL^{-1}$) and malic acid values are higher in the first ones (mean: $2,1545gL^{-1}$) and lower in the last ones (mean: $0,291gL^{-1}$). This may occur due to the malolactic fermentation process, which doesn't occur in the first ones but it does happen in the last ones. Succinic acid values are greater in red wines and citric acid values

lower. Acetic, oxalic, maleic and tartaric acid are nearly in the same concentration range between wines.

Relatively to organic acids after application of the simulated digestion model, it was not possible to detect some of them after the intestinal phase and also after gastric phase on red wine. The following table shows the variation of concentration of each organic acid compared to inicial and after gastric phase values.

Table 5. Variation of concentration of each organic acid towards the initial (⬆⬇) and after gastric phase (⬆⬇).

		LACTIC	OXALIC	SUCCINIC	MALEIC	MALIC	TARTARIC	CITRIC
VW	I	0,215	0,524	0,250	0,064	1,790	1,331	0,380
	FG	⬆	⬆	⬆	⬆	⬇	⬇	⬇
	FI	⬆/⬆	⬆/⬆	⬇/⬇	-	⬇/⬇	-	-
WW	I	0,241	0,595	0,268	0,056	1,155	1,207	0,450
	FG	⬆	⬆	⬆	⬆	⬇	⬇	⬇
	FI	⬆/⬆	⬆/⬇	⬇/⬇	-	-	-	-
RW	I	1,368	0,576	0,732	0,050	0,304	1,386	0,138
	FG	-	-	-	-	-	-	-
	FI	⬇	⬇	-	-	-	-	-

Bigger acids (with more complex structure) disappeared in the final of digestion, but simpler acids, like lactic or oxalic, increased their concentration after this process, suggesting that precipitation may occur due to pH variations[19] or bigger acids can degrade into smaller ones. However, this does not happen in the case of red wine, being the one with lower total concentration of organic acids.

Its possible to see that the concentrations of the organic acids that are described to induce an increment in gastric response[11, 13] decreased during the digestion process, apart from maleic acid, which augments but to a very low concentration (0,105gL^{-1}, Table 4). Attention should be paid to malic acid as it disappears in all wines except in Vinho Verde, after the intestinal phase. We can not say with certainty that this amount is not enough to increase gastric secretion, but in another study, even an about 6 times bigger amount didn't make a pronounced diference in gastric secretion.[13]

Since organic acids are used by intestinal microbiota for fermentation processes[12, 19, 20], that can consume and/or produce these acids, and there is no bacterial activity present in the digestion model used, this decrease can be explained by influence of pH, as referred above.

Conclusions

After this study, we can conclude that, specially after the digestive process, the differences between Vinho Verde and the other wines do not seem enough to induce different effects on gastric acidity. Even if they have initially higher concentrations of some organic acids that can stimulate gastric responses according to some studies, these considerably decrease or even disappear during digestion. This means that the fraction of organic acids that becomes available for intestinal cells to absorb is nearly the same in each type of wine, so it won't produce distinct gastric responses. Additionally, more studies can be performed to evaluate the effect of intestinal microbiota in these organic acids metabolization and to verify if the bioavailable fractions alter with the introduction of this new element.

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Attachments

Attachment A - Distillation protocol

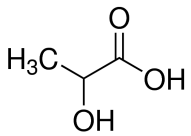
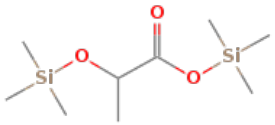
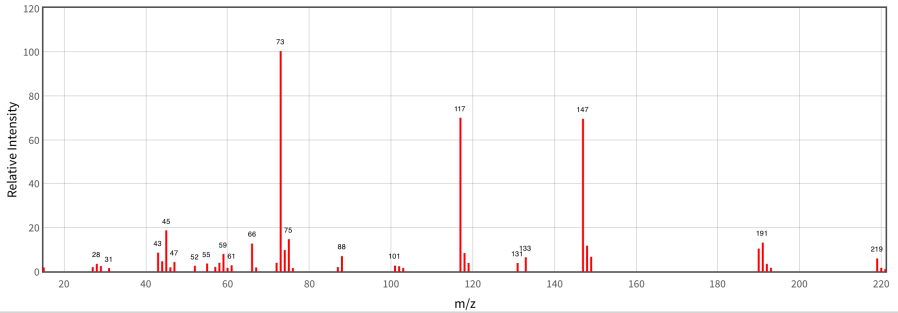
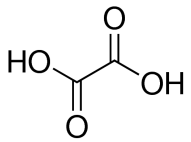
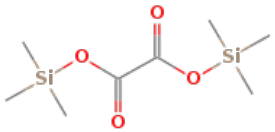
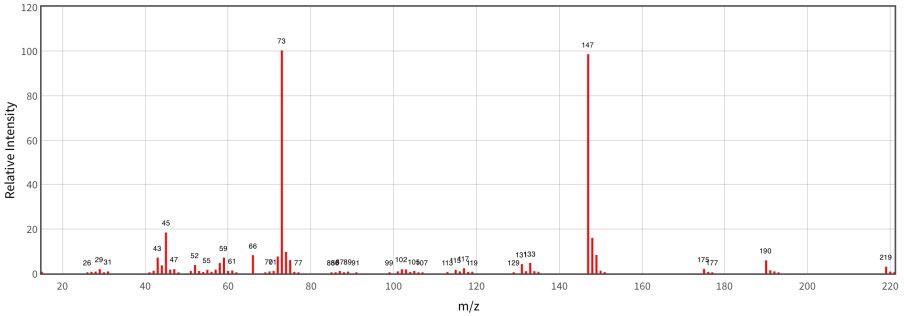
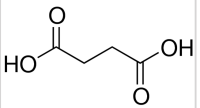
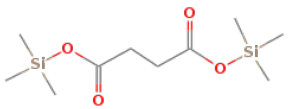
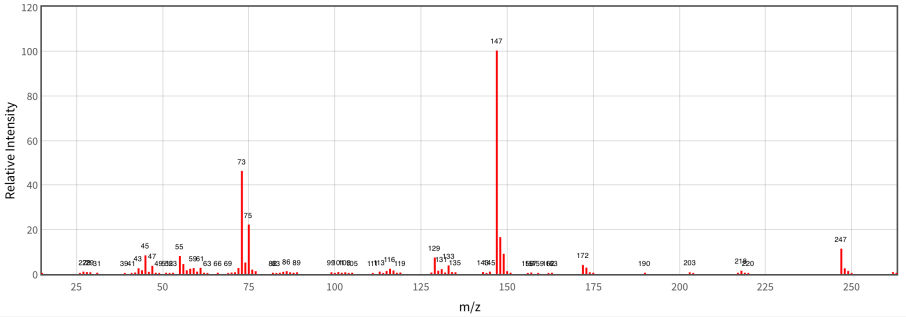
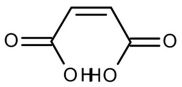
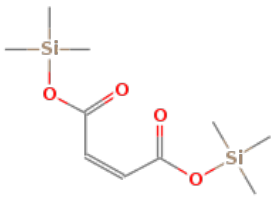
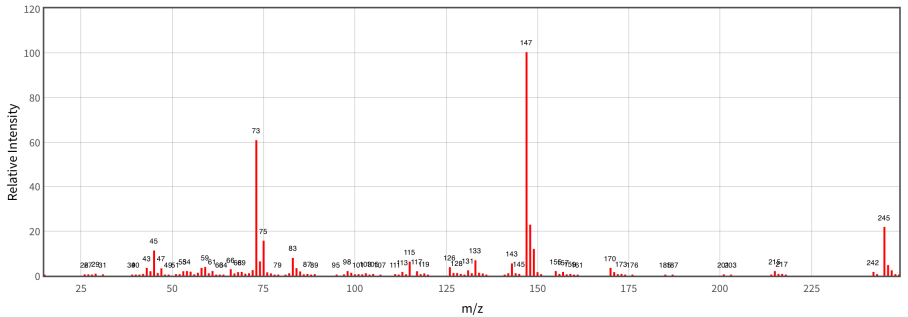
Sample: **Wine**

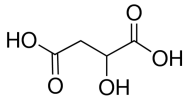
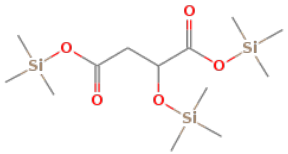
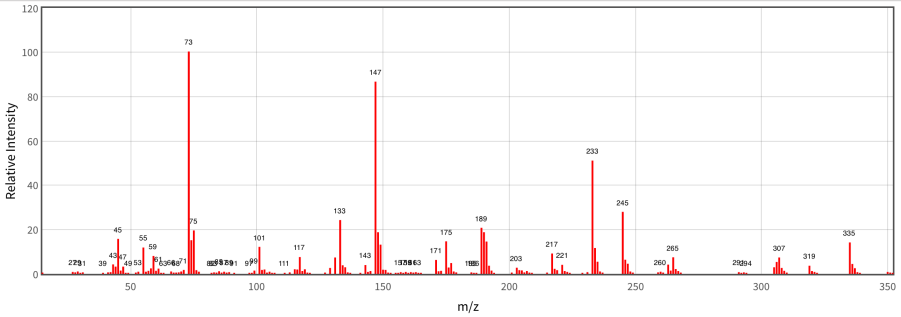
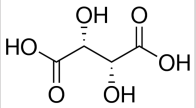
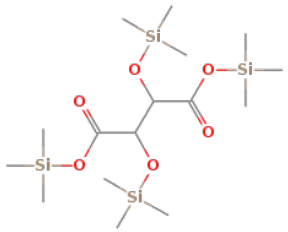
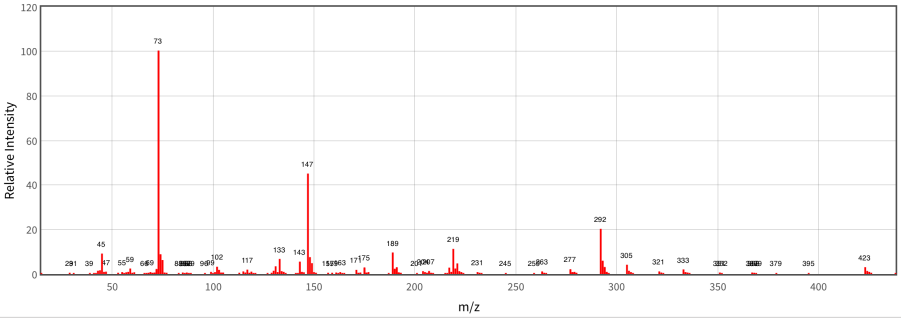
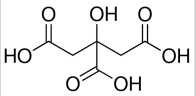
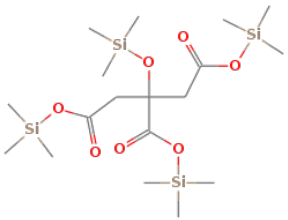
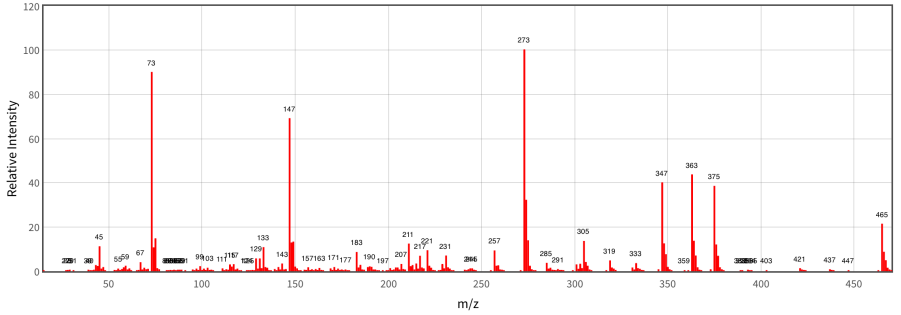
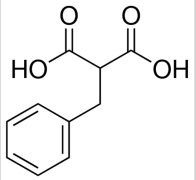
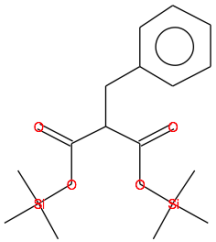
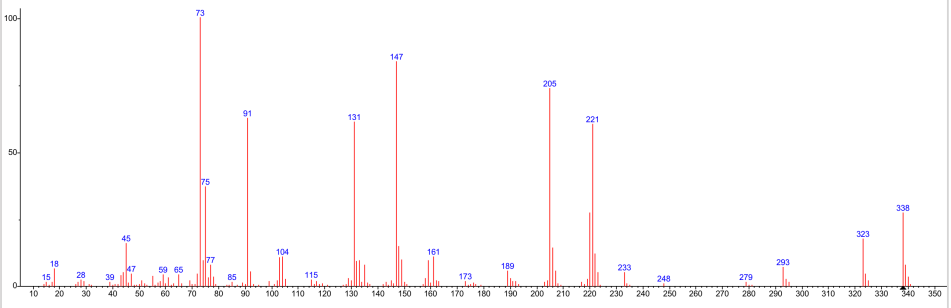
The alcohol content of a wine is defined as % (v/v) of ethanol content. Its determination is made by distillation of an alkaline sample followed by the measurement of the alcoholic strength of the distillate by areometry.

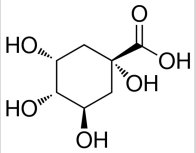
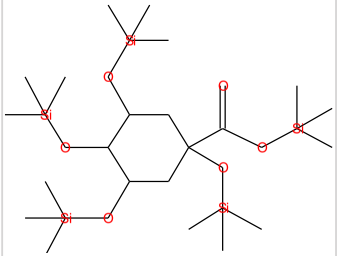
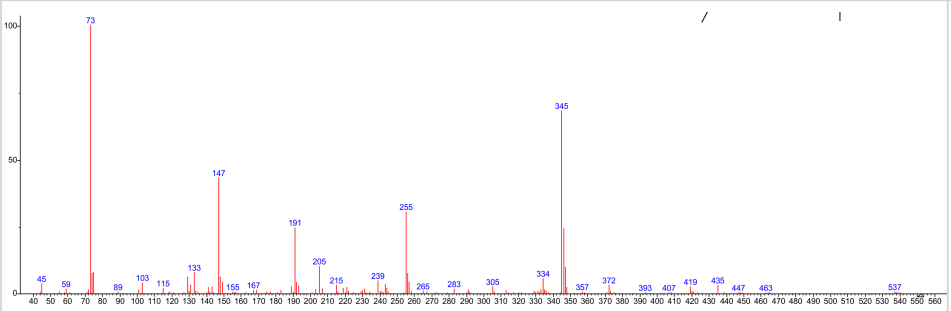
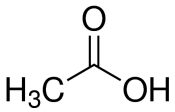
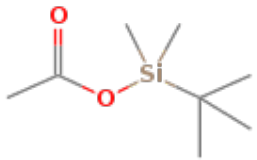
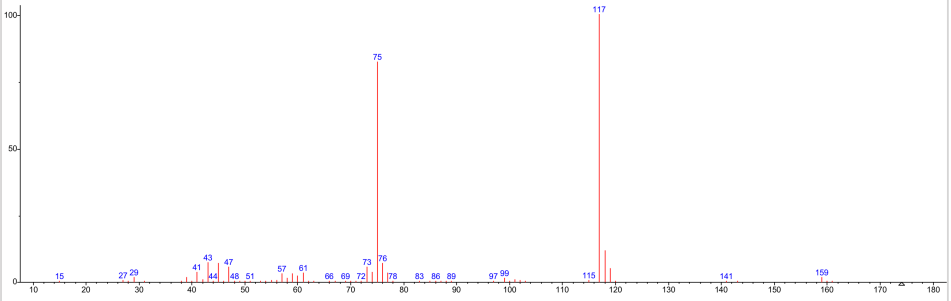
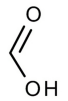
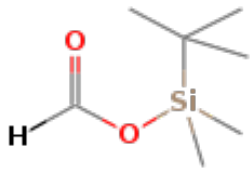
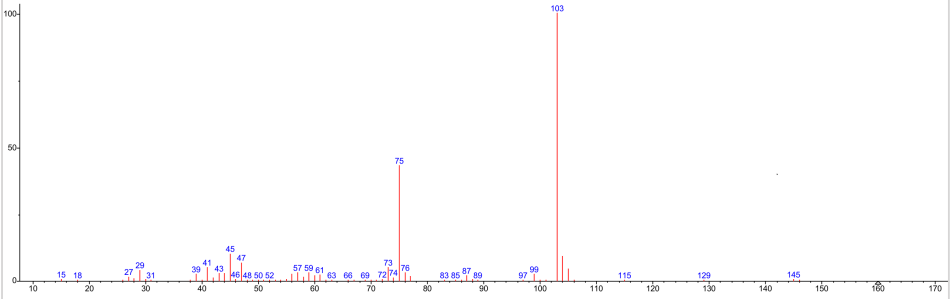
Procedure

1. Prepare a sufficient amount of sample (CO_2 elimination).
2. Fill a volumetric flask of 250mL with the drink. Note the temperature.
3. Transfer to the distillation apparatus flask, washing the flask 4 times with 5 ml of water at a time.
4. Add calcium hydroxide (2M) until alkalinization (about 10mL).
5. Heat to boiling and collect about 150mL of distillate in the same flask that was used to measure the sample (and where about 10mL of water were previously added).
6. Make up the volume until 250mL with water at the same temperature as the sample was measured.
7. Introduce the distillate into a 250mL measuring cylinder, pouring it against the wall to prevent the formation of air bubbles. Check temperature.
8. Introduce the appropriate alcoholometer and read the apparent alcohol content.
9. If the reading is at a different temperature for which the alcoholometer is graduated, make the appropriate table correction.

Attachment B - ***Organic Acids: molecular structures, characteristic ions and retention times***

Organic Acid	Molecular Structure	Molecular Structure after dervatization	Mass Spectrum	Ions	RT (min)
Láctico				117 147 191 219	6.04
Oxálico				147 148 190 219	7.99
Succínico				73 147 247	13.45
Maleico				147 215 217 245	14.62

Organic Acid	Molecular Structure	Molecular Structure after dervatization	Mass Spectrum	Ions	RT (min)
Málico				147 23 335	18.86
Tartárico				189 219 292 423	22.45
Citrico				273 347 363 465	24.35
Benzilmalónico (**PI**)				91 205 221 338	23.73

Organic Acid	*Molecular Structure*	*Molecular Structure after dervatization*	*Mass Spectrum*	*Ions*	*RT (min)*
Quínico (**PI**)				191 255 345 537	24.66
Reagente de derivatização/ Método de Injeção	**MSTFA (adição de grupo TMS) / Split, modo Full Scan**				
Acético				75 117	3.50
Fórmico (PI)				75 103	2.80
Reagente de derivatização/ Método de Injeção	**MTBSTFA (adição de grupo TBDMS) / Splitless, modo SIM**				